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(54) Title: METHODS FOR EVALUATING CHOLESTEROL METABOLISM AND REAGENTS THEREFOR

(57) Abstract

Two diagnostic methods for evaluating LDL metabolism in a patient are disclosed. A novel diagnostic agent for evaluating LDL metabolism comprising a ligand capable of binding LDL receptors and a fluorescent label and a diagnostic kit for evaluating LDL metabolism which utilized the specified diagnostic agent are also disclosed.

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METHODS FOR EVALUATING CHOLESTEROL METABOLISM AND REAGENTS THEREFOR

Field of the Invention

The present invention relates to the cardiovascular medical field and more particularly to methods for evaluating cholesterol (lipoprotein) metabolism and screening patients who have a genetic predisposition for atherosclerosis.

Background of the Invention

Atherosclerosis is the underlying cause of the majority of cardiovascular disease related deaths in the Western Hemisphere. The clinical effects of atherosclerosis result from the formation of plaque and blood clots within the lining of blood vessels which lead to arterial stenosis. Atherosclerosis at its worst has debilitating effects on blood flow to critical organs of the body and is the major cause of heart attacks and strokes in patients. Attempts to alleviate or reduce the etiology of atherosclerosis have been met only modest clinical success.

While several risk factors have been linked to the disease, studies have shown that an elevated serum cholesterol level is one of the main causes of atherosclerotic plaque formation. Cholesterol itself does not exist in a free-form in the circulation, but rather in macromolecular forms of low density lipoproteins (LDL) and high density lipoproteins (HDL). Modified (oxidized or acetylated) LDL is the harmful moiety of cholesterol. Plaque formation results when the homeostasis of lipid metabolism is unbalanced leading to an excess of modified

LDL. Recently, it has been found that hypercholesterolemia (elevated blood cholesterol levels) is not solely related to dietary intake of cholesterol. Regulation of serum cholesterol levels is controlled by three variables: dietary intake, endogenous production, and cellular metabolism.

Of these three, cellular metabolism is the most important variable in regulating serum cholesterol levels. This inducible system is regulated in most individuals with elevated serum cholesterol levels. It is believed that the clinical management of certain patients having elevated cholesterol levels would be improved if their cellular metabolism for cholesterol could be evaluated in a comprehensive manner. Thus, a need exists for diagnostic methods for evaluating cholesterol metabolism in patients.

Summary of the Invention

It has been found that cholesterol metabolism can be evaluated by monitoring the interactions of low density lipoproteins (LDL) with receptors on blood derived cells from a patient. The invention further provides a novel diagnostic agent for evaluating LDL metabolism comprising a ligand capable of binding LDL receptors and a fluorescent label and a diagnostic kit for evaluating LDL metabolism which utilizes the specified diagnostic agent.

The first diagnostic method for evaluating LDL metabolism in a patient comprises the steps of (a) contacting blood derived cells from the patient with a labeled ligand capable of binding LDL receptors; (b) incubating the resulting mixture from step (a) under conditions which allow cellular binding of the ligand and

inhibit cellular internalization of the ligand; and (c) evaluating cellular binding of the labeled ligand.

The second diagnostic method for evaluating LDL metabolism in a patient comprises the steps of (a) contacting blood derived cells from the patient with a labeled ligand capable of binding LDL receptors; (b) incubating the resulting mixture from step (a) under physiological conditions for a period sufficient to allow cellular internalization of the ligand-receptor complex; and (c) evaluating cellular internalization of the labeled ligand. In one embodiment, the blood derived cells are incubated in a cholesterol-free medium prior to step (a) to derepress (upregulate) expression of LDL receptors on the blood derived cells.

Brief Description of the Figure

Figure 1 shows the results of binding labeled LDL to white blood cells (WBCs) in two normal (1,2) and one abnormal (3) patient. Bar graph 1A and 2A indicate that the amount of total LDL uptake (37°) is approximately three times the binding ability at 4°C. Bar graph 1B and 2B indicate a 400% increase in receptor number resulting from receptor upregulation following incubation in lipid-free medium. Bar graph 3A indicates that, although receptor number appears increases in the abnormal patient, uptake is proportionately less. Bar graph 3B shows little significant upregulation of receptor number.

Detailed Description of the Invention

The present invention provides diagnostic methods for monitoring cholesterol metabolism and screening for individuals who have an elevated risk of developing atherosclerosis. The methods monitor the number of LDL

receptors on blood derived cells, specifically white blood cells, the rate of LDL and receptor internalization, and the rate of synthesis of LDL receptors. In preferred embodiments, the present methods for evaluating of LDL metabolism employ a novel diagnostic agent comprising a ligand capable of binding LDL receptors and a fluorescent label.

In the first method of the invention, blood derived cells from a patient are contacted with a labeled ligand capable of binding LDL receptors and the resulting mixture is incubated under conditions which allow cellular binding of the ligand and inhibit cellular internalization of the ligand. Suitable sources of the blood derived cells include EDTA (ethylenediamine tetraacetic acid) or herapin anti-coagulated whole blood or WBCs isolated from whole blood in accordance with conventional technique. The preferred source of blood derived cells in ACD (sodium citrate, citric acid, dextrose) anti-coagulated whole blood and the most preferred source is CPD (citrate, phosphate, dextrose) anti-coagulated whole blood. Preferably, the blood derived cells have a white blood cell concentration of from about 5×10^5 WBCs/mL to about 4×10^6 WBCs/mL and, most preferable, a concentration of from about 1×10^6 WBCs/mL to about 5×10^6 WBCs/mL.

Suitable labeled ligands are capable of binding LDL receptors and include LDL purified from a biological source and antibodies (or fragments thereof) specific for LDL receptors. Preferably, LDL is purified from whole blood of human, bovine, canine, avain, equine or porcine origin and, most preferably, from human or porcine origin. Suitable labels for the ligand include fluorescent lipid dyes, and radiolabels. Preferably, labeled

antibodies are employed at a concentration of about 10ug protein/mL to about 40ug protein/mL and labeled LDL is employed at a concentration of about 0.5 ug protein/mL to about 200ug protein/mL. Most preferably, labeled LDLis employed at a concentration of from about 10ug protein/mL to about 20ug protein/mL.

Suitable buffers employed during the incubation for binding labeled ligand to the LDL receptors include most organic and ionic buffers or phosphate buffered saline (PBS) having a pH of from about 7.2 to about 8.0 and preferably from about 7.4 to about 7.5. Preferred buffers are phosphate, bicarbonate, HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), and Tris (hydroxy-methylaminomethane) and the most preferred buffer is Hank's balanced salt solution (HBSS) supplemented with 2mg bovine serum albumin/mL and 2mM calcium at pH 7.4. Preferably, the buffer is supplemented with calcium in an amount of from about 1mM to about 3mM and bovine serum albumin (BSA) in an amount of from about 1mg/mL to about 5mg/mL.

Preferably, the incubation is conducted for a period of from about 30 minutes to about 20 hours and, most preferably, for a period of about 2 hours. Preferably, cells should be preincubated at the appropriate temperature for about 15 minutes prior to the addition of the labeled ligand. Suitable temperatures for the incubation are from about 1°C to about 15°C and, preferably, from about 1°C to about 4°C. Following incubation, cellular binding of the labeled ligand-receptor composition is evaluated. The method employed for evaluating cellular binding is dependent on the selected label. For example, in a preferred embodiment, the label is a fluorescent dye and the cellular binding of the labeled ligand is

evaluated in a flow cytometer employing conventional techniques. Suitable methods evaluating cellular binding may also include monoclonal antibodies labeled with a fluorescent dye or radiolabeled LDL or monoclonal antibodies. Cellular binding may suitably be evaluated with epifluorescent microscopy or equipment to detect radiolabel employing conventional techniques.

In the second method of the invention blood derived cells from a patient are contacted with a labeled ligand capable of binding LDL receptors and the resulting mixture is incubated under physiological conditions for a period sufficient to allow cellular internalization of the labeled ligand-receptor complex. Suitable and preferred sources of blood derived cells, labeled ligand, and buffers are as specified above. Preferable, cells should be preincubated at the appropriate temperature for about 15 minutes prior to the addition of the labeled ligand. The incubation of the cells and ligand is conducted for a period of from about 30 minutes to about 20 hours and, most preferably, for about 2 hours. Suitable temperatures are from about 20°C to about 40°C and, preferably, from about 30°C to about 38°C. Following incubation, cellular internalization of the labeled composition is evaluated. In a preferred embodiment, the internalization of the labeled ligand-receptor composition is evaluated in a flow cytometer employing conventional techniques.

In one embodiment of the above method, the blood derived cells are pretreated by incubation in a cholesterol-free medium to derepress expression of LDL receptors on the blood derived cells. It has been found that individuals with hypercholesterolemia, either heterozygous or homozygous, have little or no ability

respectively, for either producing or internalizing LDL receptors in their cells and individuals with pathological diseases such as diabetes and thyroiditis exhibit various defects in lipid metabolism. Also, normal individuals who consume excessive amounts of cholesterol suppress normal LDL receptor production.

It has been found that one method for distinguishing true genetic or pathological conditions from normal individuals is to incubate cells in a cholesterol-free medium. The incubation in cholesterol-free medium allows for new receptors to be synthesized and expressed in the normal individual. It has been found that individuals with genetic defects exhibit little or no ability to express this upregulation of receptors. Any complete cell culture minimal medium is suitable for the derepression incubation. The most preferred medium is RPMI 1640 supplemented with 5% lipid free fetal bovine serum and 10ug insulin/mL. Preferably, the pretreatment is conducted at a temperature of from about 35°C to about 40°C and for a period of from about 18 hours to about 72 hours. The most preferred period for derepression incubation is from about 20 hours to about 24 hours.

The invention also provides a diagnostic agent for use in the first and second methods described above which comprises a ligand capable of binding LDL receptors and a fluorescent label. As stated above, suitable ligands include LDL purified from a biological source and antibodies specific for LDL receptors.

Preferably, LDL is purified from whole blood human, bovine, canine, avain, equine or porcine origin and, most preferably, from human or porcine origin.

In this preferred embodiment, the ligand is labeled using conventional techniques with fluorescent dye.

Preferred labels for LDL are selected from the group consisting of DiI (1,1'diocta-decyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), DiO(3,3'dioctadecyloxacarbocyanine perchlorate), and S-467 (N-[3-sulfopropyl]-4-[p-di-decylaminostyryl]pyridinium). Advantages associated with the specified labels include excitation in a range achievable by the vast majority of flow cytometers. DiO can be combined with DiI for double label experiments of diagnostic tests (i.e., LDL, HDL receptors assay ratios), DiO has a sharp emission peak that does not overlap with DiI. The advantage of S-467 is the intensity of its emission making quantitative results very sensitive.

Monoclonal antibodies specific for LDL receptors are produced by antibody-producing cell lines which may be hybrid cell lines commonly known as hybridomas. The hybrid cells are formed by the fusion of an anti-LDL receptor antibody-producing cell and an immortalizing cell line. In the formation of the hybrid cell lines, the first fusion partner - the anti-LDL receptor antibody-producing cell - may be a spleen cell of an animal immunized against a LDL receptor positive T cell or a biological preparation comprising LDL receptor. Alternately, the anti-LDL receptor producing cell may be a B lymphocyte obtained from the spleen, lymph nodes or other tissue. The second fusion partner - the immortal cell - may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody-producing cell but also malignant.

Murine hybridomas which produce LDL receptor specific monoclonal antibodies are formed by the fusion of mouse myeloma cells and spleen cells from mice immunized against a LDL receptor positive T cells,

purified LDL receptor, or other biological preparations comprising LDL receptor. To immunize the mice, a variety of different protocols may be followed. For example, mice may receive primary and boosting immunizations of LDL receptor positive T cells. The fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Kohler and Milstein, Nature, 256:495-497 (1975) and Kennet, Monoclonal Antibodies (Kennet et al., eds. pp. 365-367, Plenum Press, N.Y., 1980).

The resulting clones are then screened for production of antibody reactive with LDL receptor positive T cells or biological preparations comprising LDL receptor. Those which secrete reactive antibodies are cloned and the desired monoclonal antibody is purified from said clones using conventional immunological techniques.

The present invention is further described by the following examples wherein all parts and percentages are by weight and degrees are Celsius.

EXAMPLE

A. Procedure for testing functional activity of low density and/or high density lipoprotein receptors in human mononuclear cells.

I. Isolation of Lipoproteins and Fluorescence Labeling.

Porcine LDL was isolated from whole blood obtained from a local abattoir. 1000mL of whole blood was combined with 10mL of EDTA (10%) at the time of collection. The LDL and HDL components of the whole

blood were isolated according to procedures described in Cancer Research, 43:4600-4605 (1983) and J. Lipid Research, 20:217-229 (1979). Whole blood was centrifuged at 5,000 x g for 30 minutes at 4°C to remove the cellular components. The density of the plasma was increased to 1.019g/mL with KBr. Samples were ultracentrifuged at 300,000 x g for 30 hours at 4°C. The lipoprotein layer was removed and the density adjusted to 1.065g/mL with KBr. Samples were again ultracentrifuged at 300,000 x g for 40 hours at 4°C. LDL and HDL bands were removed. The individual components were dialyzed against 0.9% NaCl 0.3 mM EDTA pH 7.4 to remove the KBr. Samples were stored at 4°C.

The resulting purified lipoproteins were fluorescently labeled with DiI, DiO, or S-467 according to the method described in J. Cell Biology, 90:595-604 (1981). Two (2) mg of lipoprotein solution were combined with 25 mg of insoluble potato starch, frozen in liquid nitrogen and lyophilized. Lyophilized samples were mixed with 2 mg of the fluorescent dye dissolved in 0.5mL methanol, and placed at 4°C for 2 hours. The mixture was evaporated to dryness under nitrogen at 4°C. One mL of buffer, 0.12M NaCl, 10 mM Tricine pH 8.2, was added. The mixture was incubated 41 hours at 4°C. Starch was removed via centrifugation at 4,000 x g for 20 minutes at 4°C. The supernatant containing the buffer and suspended labeled lipoproteins was removed and stored at 4°C protected from light.

II. Patient Blood Collection.

Ten (10)mL samples of whole blood were collected from patients via venapuncture into a ACD solution A

vacutainer tube (yellow top). Immediately following collection, 1.5mL of blood was divided into three (3) 0.5mL aliquots and placed into 50 mL conical tubes. The aliquots and placed into 50mL conical tubes. The aliquots were diluted to a final volume of 50mL with HBSS without calcium. It has been found that initial washes with calcium-free medium reduce clotting, helps preserve the lipoprotein receptors and removes nascent, blood-borne LDL. The tubes were centrifuged for 8 minutes at 350 x g at 22-24°C. The resulting wash supernatants were discarded and the pellets are resuspended to a final volume of 50mL with HBSS without calcium and recentrifuged as described above. The resulting pellets were resuspended in 2mL HBSS and supplemented with 2mg/mL BSA and 2mM $CaCl_2$ at pH 7.4.

III. Incubation of Fluorescent Lipoproteins with Blood Derived Samples.

The three blood samples, described above and designated A, B, and C hereinafter, were maintained at the following temperatures: A and B at 4°C and C at 37°C. Cells were preincubated at the appropriate temperature for 15 minutes prior to the addition of the labeled ligand. Fifteen (15) ug of the labeled lipoprotein described above were added to B and C, and A was maintained as a control for determining autofluorescence. All three samples are incubated for two hours. The resulting samples were then fixed by the addition of 5mL of 4% formaldehyde in PBS at 4°C for 5 minutes and then for five minutes at ambient temperature. It was discovered that if lyzing of RBCs (red blood cells) precedes fixation, the assay does not yield

consistent results and that if cells are fixed for a shorter period of time, fixation is inadequate while longer periods of fixation reduce the ability to lyse RBCs.

Lyzing of RBCs to completion was accomplished by adding 15mL of deionized water at 4°C. As soon as RBC lysis is complete, the volume of each sample was brought up to 50mL with PBS supplemented with 5% BSA to prevent WBC lysis from occuring. Analysis may also be completed without lysis of RBCs. The resulting samples were washed twice by centrifugation for eight minutes at 350 x g at ambient temperature. (Unbound fluorescent lipoproteins remain in solution and are discarded with the supernatant.) The final pellets were resuspended in 0.5mL PBS supplemented with 5% BSA. It has been found that the resulting samples are stable at 4°C for five days if maintained in a light-free environment.

IV. Derepression Assay (LDL Receptor Upregulation)

An additional 1.5mL of the original 10mL of the whole blood drawn from the patient were divided into 0.5mL aliquots and washed as outlined in II. The final pellets were suspended in tissue culture flasks with 30mL of RPMI 1640 medium supplemented with 5% lipid-free fetal bovine serum and 10ug/mL insulin. The resulting cells were incubated for 24 hours at 37°C to maintain cell respiration and viability. The cells were centrifuged and then washed in HBSS as outlined in II. Fluorescent LDL or HDL binding is performed as outlined in III.

It has been found from studies involving the blood of twenty normal patients that LDL receptors increase up to about 400% following incubation in lipid-free medium

for 24 hours. Preliminary data for people with various pathologies indicate that the upregulation effect for the most part, is far less dramatic.

V. Results of Histogram Comparisons.

Relative number of receptors (S_1), or binding at 4°C, is expressed as the difference in intensity of fluorescence (channel number) between the unlabeled control and sample labeled at 4°C. Differences are read for 95% of the cell population. Uptake, or ability of receptors to internalize LDL into the cell, (S_2), is expressed as the difference in the intensity of fluorescence between the sample labeled at 4°C and the samples labeled at 37°C. As shown in Figure 1, the ratio S_1/S_2 in normal individuals is approximately 1:3 to 1:4.

The rate of synthesis of new receptors is measured as the ratio between S_1 from freshly labeled cells and S_1 from the derepressed cells. For normal individuals, the range is approximately 1:4. The relative number of receptors and the uptake of LDL, along with derepression, will yield information reflecting the physiological status, as pertaining to lipoprotein metabolism, in patients tested.

CLAIMS

1. A method for evaluating LDL metabolism in a patient comprises the steps of:

a) contacting blood derived cells from the patient with a labeled ligand capable of binding LDL receptors;

b) incubating the resulting mixture from step (a) under conditions which allow cellular binding of the ligand and inhibit cellular internalization of the ligand; and

c) evaluating cellular binding of the labeled ligand.

2. A method according to Claim 1, wherein the labeled composition is labeled LDL.

3. A method for evaluating LDL metabolism in a patient comprises the steps of:

a) contacting blood derived cells from the patient with a labeled ligand capable of binding LDL receptors;

b) incubating the resulting mixture from step (a) under physiological conditions for a period sufficient to allow cellular internalization of the ligand-receptor complex; and

c) evaluating cellular internalization of the labeled ligand.

4. A method according to Claim 3, wherein the blood derived cells are incubated in a substantially cholesterol-free medium prior to step (a).

5. A method according to Claim 3, wherein the labeled composition is labeled LDL.

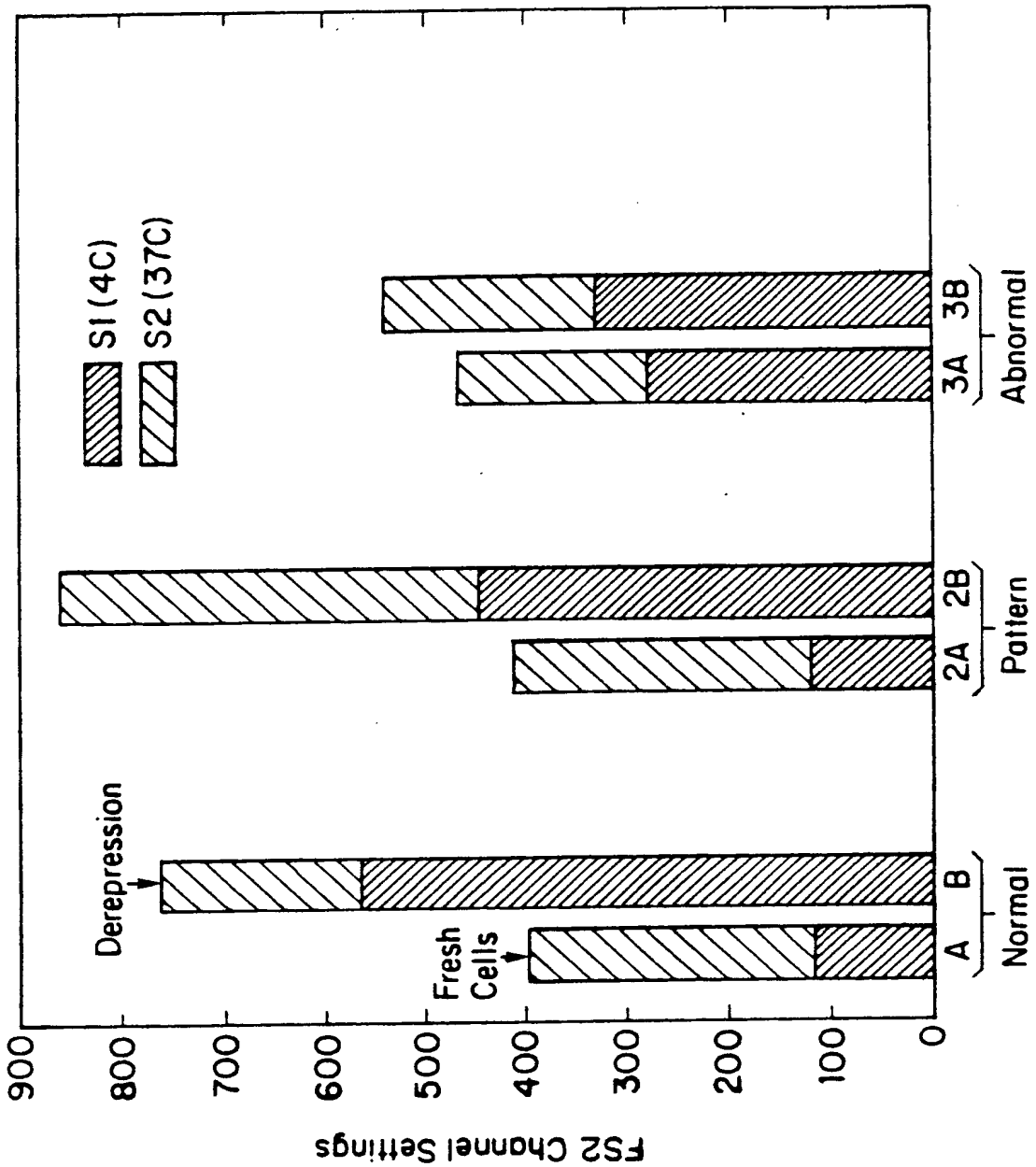
6. A novel diagnostic agent for evaluating LDL metabolism comprising substantially purified porcine LDL or HDL and a fluorescent label.

7. A kit for evaluating LDL metabolism comprising the diagnostic agent of Claim 6.

8. A method according to Claim 1, wherein the labeled composition is a labeled antibody reactive with LDL receptors.

9. A method according to Claim 3, wherein the labeled composition is a labeled antibody reactive with LDL receptors.

Fig. 1



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No [redacted] US 90/06123

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/92, 33/58

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Dialog Information Service, file 155: Medline 66-91, Medline Accession number 88120516, KN Traill et al: "Analysis of fluorescent low density lipoprotein uptake by lymphocytes" & Mech Ageing Dev, oct 1987, 40 (3) p261-88, abstract --	3-5,7
X	Dialog Information Service, file 155: Medline 66-91, Medline Accession number 83301118, I Melzner et al: "Radiochemical and immunohistochemical determination of surface binding of low density lipoproteins on lymphocytes" & Acta Histochem Suppl 1983, 28 p199-207, abstract --	3-8
X	Dialog Information Service, file 155: Medline 66-91, Medline Accession number 86164374, TT Chen et al: "Receptor-mediated uptake of lipoproteins by cultured porcine granulosa cells" & Eur J Cell Biol, jan 1986, 39 (2) p410-6, abstract --	3,5,6

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th February 1991	- 6 MAR 1991
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Chemical Abstracts, volume 101, no. 17, 22 October 1984, (Columbus, Ohio, US), Huettinger, Manfred et al.: "Use of monoclonal anti-receptor antibodies to probe the expression of the low density lipoprotein receptor in tissues of normaland Watanabe heritable hyperlipidemic rabbits ", see page 522, abstract 149343a, & J. Clin. Invest. 1984, 74(3), 1017-1026	8
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X	Chemical Abstracts, volume 106, no. 23, 8 June 1987, (Columbus, Ohio, US), Wojciechowski, Andrew P et al.: "Receptor-mediated endocytosis of fluorescent-probe-labelled low-density lipoprotein using human lymphocytes, fluorometry and flow cytometry ", see page 368, abstract 192182n, & Biochem. Soc. Trans. 1987, 15(2), 251- 252	3,5,7
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A	Dialog Information Service, file 5: BIOSIS 68-91: Biosis Accession number 80018974, MJ Rudling et al: "A simple binding assay for the determination of low-density lipoprotein receptors in cell homogenates" & Biochimica et Biophysica Acta 833 (3), 1985, 359-365, abstract	1,2,9
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